Pediatr Surg Int (2013) 29:249–256
DOI 10.1007/s00383-013-3268-x

ORIGINAL ARTICLE

Optimizing in vitro culture conditions leads to a significantly shorter production time of human dermo-epidermal skin substitutes

Luca Pontiggia · Agnieszka Klar · Sophie Böttcher-Haberzeth · Thomas Biedermann · Martin Meuli · Ernst Reichmann

Accepted: 16 January 2013 / Published online: 3 February 2013
© Springer-Verlag Berlin Heidelberg 2013

Abstract
Introduction Autologous dermo-epidermal skin substitutes (DESS) generated in vitro represent a promising therapeutic means to treat full-thickness skin defects in clinical practice. A serious drawback with regard to acute patients is the relatively long production time of 3–4 weeks. With this experimental study we aimed to decrease the production time of DESS without compromising their quality.
Methods Two in vitro steps of DESS construction were varied: the pre-cultivation time of fibroblasts in hydrogels (1, 3, and 6 days), and the culture time of keratinocytes (3, 6, and 12 days) before transplantation of DESS on nude rats. Additionally, the impact of the air–liquid interface culture during 3 days before transplantation was investigated. 3 weeks after transplantation, the macroscopic appearance was evaluated and histological sections were produced to analyze structure and thickness of epidermis and dermis, the stratification of the epidermis, and the presence of a basal lamina.
Results Optimal DESS formation was obtained with a fibroblast pre-cultivation time of 6 days. The minimal culture time of keratinocytes on hydrogels was also 6 days. The air–liquid interface culture did not improve graft quality.
Conclusion By optimizing our in vitro culture conditions, it was possible to very substantially reduce the production time for DESS from 21 to 12 days. However, pre-cultivation of fibroblasts in the dermal equivalent and proliferation of keratinocytes before transplantation remain crucial for an equilibrated maturation of the epidermis and cannot be completely skipped.

Keywords Tissue engineering · Dermo-epidermal skin substitutes · Skin reconstruction · Air-liquid interface · Collagen hydrogels

Introduction

Cultured skin substitutes have been used in both experimental and clinical settings for over 40 years [1]. In the global picture, culture time was always between 3 and 4 weeks, independent of what type of skin substitute was produced [cultured epithelial autografts (CEA) alone [2–5], CEA + allografts [6]], or cultured skin substitutes (CSS) [7, 8].

For obvious reasons, culture time does not play a dominant role when a cultured skin substitute is transplanted onto a patient undergoing elective plastic or reconstructive surgery since the patient can be called in for the operation when the graft is ready. In contrast, culture time does play a crucial, potentially even vital, role when cultured skin substitutes are to be applied on acute and massive burn patients. Therefore, a significant reduction of culture time would represent a substantial progress. We hypothesize that a reduction of pre-cultivation times of both fibroblasts and keratinocytes could substantially shorten the production time without negative effects on graft quality. Here, we present a culture modality to tests the above hypothesis.

L. Pontiggia and A. Klar contributed equally to this paper.

L. Pontiggia · A. Klar · S. Böttcher-Haberzeth · T. Biedermann · E. Reichmann
Tissue Biology Research Unit, University Children's Hospital Zurich, Zurich, Switzerland

S. Böttcher-Haberzeth · M. Meuli (✉)
Department of Pediatric Surgery, University Children's Hospital Zurich, Steinwiesstrasse 75, 8032 Zurich, Switzerland
e-mail: martin.meuli@kispi.uzh.ch

Materials and methods

Primary cell cultures

Human skin samples from scalp, eyelid, neck or foreskins were obtained from patients aged between 4 months and 56 years (Table 1). Approval was obtained from the Ethics Committee of the Canton Zurich and informed consent was given by parents or patients. Keratinocytes and fibroblasts were isolated, cultured, and stored in liquid nitrogen until needed as previously described [9].

Table 1 Skin samples

Series	Cell type	Donor site	Donor age	Sex	Passage
Phase I: preincubation of fibroblasts					
1	Fibroblasts	Neck	2 years	Male	P1
2	Fibroblasts	Foreskin	8 years	Male	P1
3	Fibroblasts	Foreskin	4 years	Male	P2
1,2,3	Keratinocytes	Foreskin	8 years	Male	P1
Phase II: preincubation of keratinocytes					
1,2	Fibroblasts	Eyelid	45 years	Female	P1
1	Keratinocytes	Eyelid	56 years	Male	P1
2	Keratinocytes	Eyelid	2.5 years	Female	P1

Organotypic cultures and transplantation of cultured dermo-epidermal composites: standard procedure

Organotypic cultures and transplantation experiments were performed as previously published [9] with some modifications: hydrogels were prepared by mixing 0.6 ml chilled neutralization buffer containing 0.15 M NaOH [10], 0.3 ml DMEM/10 % FCS containing 40,000 fibroblasts and 2.1 ml of rat tail collagen type I (3.2–3.4 mg/ml, BD Biosciences, Allschwil Switzerland) into 4.2 cm^2 cell culture inserts (BD Falcon, Basel, Switzerland [11]). After jellification (10 min at room temperature and 2 h at 37 °C) the thickness of the dermal equivalents was reduced from 7 to 1 mm by compression [12] and grown in DMEM/10 % FCS for 1–6 days. Subsequently, keratinocytes were seeded onto each dermal equivalent at a density of 125×10^3 cells/cm^2 within polypropylene rings of 5 mm in diameter. After 6 h the rings were removed and culture medium was

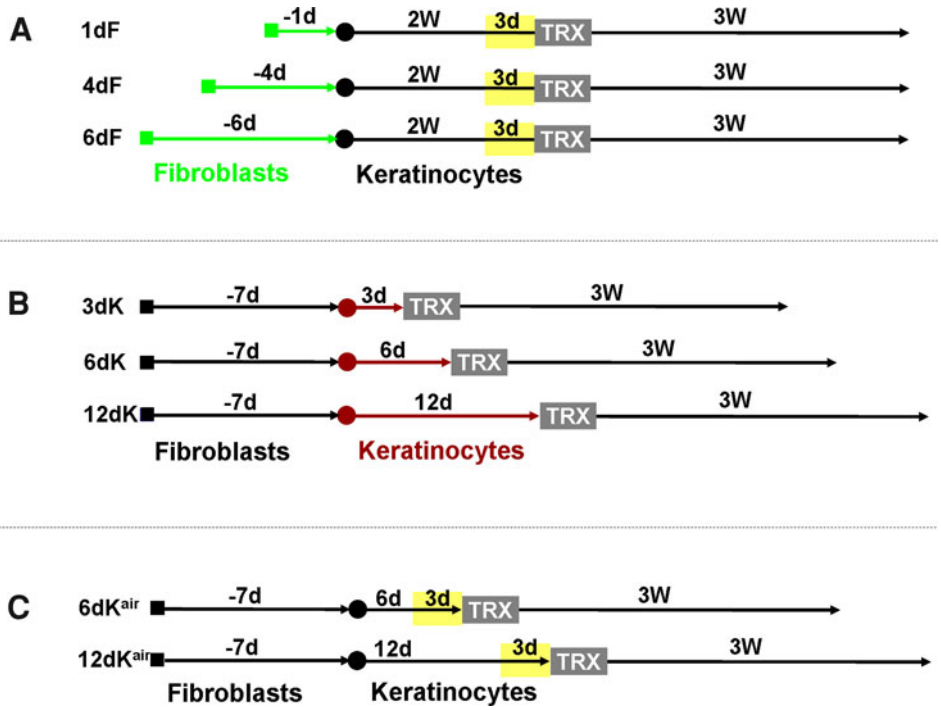

Fig. 1 Schematic representation of the experimental design. We evaluated modifications of our standard protocol for the in vitro DESS construction in three steps. **a** In the first part of the study, fibroblasts were included in collagen I hydrogels and cultivated (*green line*) for 1 (1dF), 4 (4dF), and 6 (6dF) days before being covered by a keratinocyte sheet (*black dot*). After 2 weeks (including air–liquid culture for 3 days), DESS were transplanted (TRX) on nude rats and grafts were removed for analysis 3 weeks post transplantation. **b** In the second part of the study, fibroblasts were included in collagen I hydrogels and cultivated for 7 days before being covered by a keratinocyte sheet (*red dot*). After further cultivation (*red line*) for 3 (3dK), 6 (6dK), and 12 (12dK) days DESS were transplanted (TRX) on nude rats and grafts were removed for analysis after 3 weeks. (**c**) A second set of 6dK and 12dK was treated in the same way as in **b**, but they were exposed to the air–liquid interface 3 days before transplantation ($6dK^{+air}$ and $12dK^{+air}$) while the original DESS remained submerged

added in the upper and lower chambers. The skin equivalents were cultured for 2 weeks; 3–4 days before transplantation on nude rats [9], the substitutes were raised to the air–liquid interface.

Variations of the standard protocol: experimental schedule

The most time-consuming steps during culturing of skin analogs are the pre-incubation times for fibroblasts and keratinocytes. Therefore, we focussed on whether shortening those periods would yield the envisioned culture time reduction. In the first experiment, the pre-incubation time of fibroblasts in the dermal template was modulated (Fig. 1a). We isolated fibroblasts from foreskin or neck skin biopsies of three different patients and cultured them to passage 1–2. The age of the patients varied between 4 months and 2 years (Table 1). 1, 4, or 6 days before the pre-determined date of keratinocytes seeding (green squares) fibroblasts were included in collagen I hydrogels. At day 0 (black dots), foreskin keratinocytes were applied onto the hydrogels and cultured for 11 days. The hydrogels were exposed to air-liquid interface 3 days before transplantation (TRX).

In the second part of the experiment, the pre-incubation time of fibroblasts in the dermal template was maintained constant at 7 days (Fig. 1a). Thereafter, at day 0 (red dots) keratinocytes were seeded on the hydrogels and incubated for 3, 6, or 12 days before transplantation. Two sets of the 6- and 12 days series were produced. One of them was exposed to the air/liquid interface 3 days before transplantation (Fig. 1c), while the other remained submerged (Fig. 1b). In all experiments, grafts were removed for analysis 3 weeks after transplantation.

Fluorescein diacetate (FdA) vital cell staining

In order to visualize cell viability and homogenous epidermal cell coverage of the hydrogels, FdA staining was performed as published [13]. Briefly, from an acetone 5-mM stock solution, FdA (Sigma, Buchs, Switzerland) was added to the culture medium in the lower and upper chambers to a final concentration of 5 μM. After 2 min, FdA was removed by washing twice in PBS before fresh culture medium was applied. The substitutes were analyzed by fluorescence microscopy.

Histology and immunofluorescence microscopy

The epidermal substitutes were prepared to produce cryo- and paraffin sections. Histology and three-color immunofluorescence stainings were performed as previously described. For details refer to Biedermann et al. [9].

Antibodies

For the purpose of determining DESS quality, antibodies to the following markers were applied for immunofluorescence stainings:

Involucrin (clone SY5, 1:100; LabVision, P.H.Stehelin&CIE AG, Basel, Switzerland) is a marker for the cornification process and is associated with the formation of desmosomes and intermediate filaments in the granular layer [14]. It is expressed (in homeostatic conditions) in the granular and cornified layer. K1 (clone LHK1, 1:200; Chemicon) as late differentiation marker [15] is indicative of the degree of tissue homeostasis in normal human skin, where it is expressed in all suprabasal layers, with exception of the stratum corneum. Occludin (polyclonal, 1:50; Zymed, Invitrogen, Basel, Switzerland) is indicative for the formation of tight junctions in the granular layer. It represents a further confirmation of acquiring terminal functionality of the epidermis. α6 integrin (clone 4F10, 1:100; Chemicon, Millipore AG, Zug, Switzerland) is a component of hemidesmosomes and assures the stable anchorage to the basal lamina [16]. It is indicative for the quality of the dermo-epidermal junction. K19 (clone RCK108, 1:100; Dako, Baar, Switzerland) and K15 (clone spm190, 1:50; Santa Cruz, Labforce AG, Nunningen, Switzerland) are

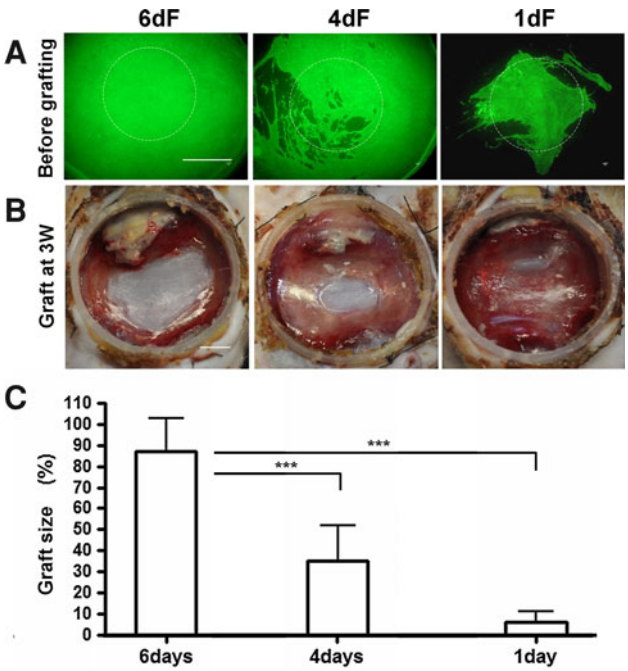

Fig. 2 Modulation of the pre-incubation time of fibroblasts in DESS: effect on graft take in vivo. **a** Fluorescein diacetate (FdA) staining of one set of DESS immediately before transplantation (*upper panels*): the *white dotted circles* denote the keratinocyte seeding area. **b** Macroscopic view of the grafts 3 weeks thereafter. *Scale bars* 5 mm. **c** Graft size 3 weeks after grafting in % of the originally transplanted DESS (mean ± SD, $n = 3$). ***$p < 0.0002$

markers for a mature basal layer and general homeostasis: in homeostatic engineered epidermal substitutes K19-positive cells are clustered in the stratum basale as a subpopulation of K15-positive keratinocytes [11]. As a secondary antibody we used FITC-conjugated polyclonal goat F(ab')$_2$ fragments directed to mouse immunoglobulins (Dako).

For double immunofluorescence, some of the primary antibodies were prelabeled with Alexa 555-conjugated polyclonal goat F(ab')$_2$ fragments, according to the instructions of the manufacturer (Zenon Mouse IgG Labeling Kit, Molecular Probes, Life Technologies, Zug, Switzerland).

Statistical analysis

The thickness of epidermis and stratum corneum as well as the graft size was measured in three different representative areas. All results are reported as mean ± standard deviation. Statistical analysis was performed with GraphPad Prism 4.0 (Graph Pad software, La Jolla, CA, USA). Comparison between two groups was performed using the unpaired Student's t test. Results were considered significant with a $p < 0.05$.

Results

Fibroblasts pre-cultivation time

Immediately before transplantation we verified the presence of an epidermis via FdA staining. Figure 2a shows the staining of the second series of substitutes (Table 1). Similar results were obtained with series one and three: keratinocyte proliferation was more pronounced on the hydrogels which were conditioned by fibroblasts for 6 days (6dF): the whole surface of the gels was homogeneously covered (Fig. 2a, left panel), starting from a circle of 0.96 mm in diameter in the center of the dermal template (dotted circle). A shorter pre-incubation of the fibroblasts

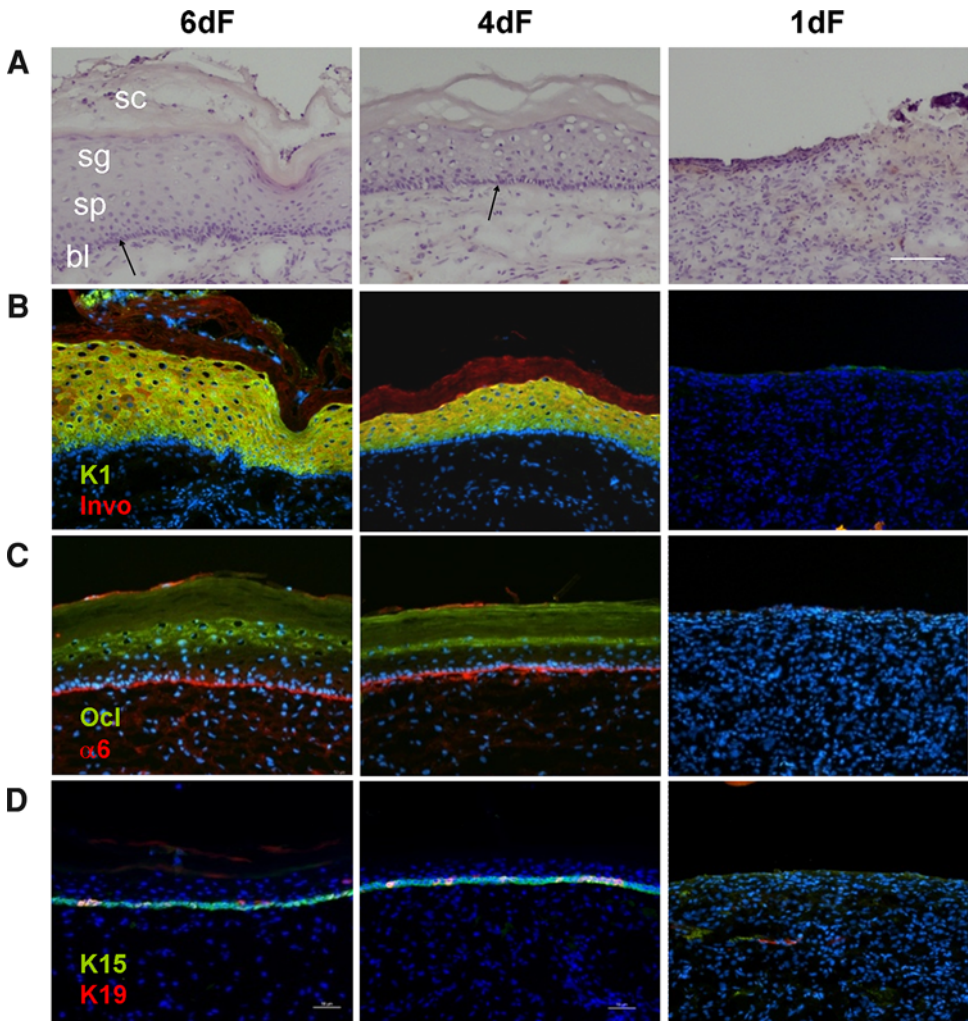

Fig. 3 Expression of structural markers for advanced epidermal histogenesis. **a** Eosin/Hematoxylin-stained paraffin sections of 6dF, 4dF, and 1dF-DESS 3 weeks after transplantation. *bl* basal layer (*arrow*), *sp* stratum spinosum, *sg* stratum granulosum, *sc* stratum corneum. **b** Immunofluorescence double staining of cryosections with antibodies against cytokeratin K1 (*green*) and involucrin (*red*), **c** occludin (*green*), and integrin α6 (*red*), or **d** cytokeratin K15 (*green*) and K19 (*red*). Scale bar for all *panels* 100 μm

of 4 days (4dF) or 1 day (1dF) was less supportive for keratinocyte proliferation (middle and right panel).

Three weeks after transplantation (Fig. 2b), the 6dF-DESS looked macroscopically more developed and the presence of the hydrophobic stratum corneum was clearly visible (left panel). Correspondingly the measurement of the graft area showed that 87 ± 16 % of the original graft size was maintained (Fig. 2c). In contrast, in 4dF and 1dF-DESS the graft area was reduced to 35 ± 17, and 6 ± 5 %, respectively (Fig. 2c).

Histological analyses confirmed in 6dF-DESS the formation of a stratified epidermis, which consisted of a stratum basale (sb), 12–20 suprabasal layers comprising stratum spinosum (ss) and stratum granulosum (sg), and a well-differentiated anuclear stratum corneum (sc, Fig. 3a). In 4dF-DESS, the epidermal thickness was reduced (middle panel). In 1dF-DESS, no epithelium was found (right panel).

Stratification markers in engineered DESS

As a complement to the morphological analysis, we verified the presence of some protein expression markers which are indicative for the grade of homeostasis of an engineered epidermal substitute.

We found involucrin expression (Fig. 3b, red stain) in the stratum spinosum of the substitutes, indicating a still ongoing differentiation process in 6dF-DESS (Fig. 3b, left). The thinner epidermis in 4dF showed a similar expression pattern (Fig. 3b, center). No involucrin expression was found in the in 1dF (Fig. 3b, right).

Both 6dF and 4dF showed a strong expression of K1 (Fig. 3b, green stain, left and center) in all suprabasal layers, with exception of the cornified envelope. In 1dF, only few K1-expressing epithelial cells were visible (Fig. 3b, right).

Occludin (Fig. 3c, green) was expressed in the upper stratum granulosum of 6dF and 4dF (left and center), but absent in 1dF (right). Integrin α6 staining (Fig. 3c, red) demarcated the dermo-epidermal junction in 6dF and 4dF, but was not visible in 1dF.

Figure 3d shows that in 6dF and 4dF, but not in 1dF, K19-positive cells (red), are present in the basal layer as a subpopulation of K15-positive keratinocytes (green).

Keratinocyte pre-cultivation time

The second step of DESS generation consisted in the seeding of keratinocytes on the pre-cultivated hydrogels. We now varied the incubation time of keratinocytes on the dermal substitutes (and so the degree of pre-stratification of the graft) before transplantation while the pre-cultivation time of the fibroblasts in the gels was maintained identical (Fig. 1b). Figure 4a illustrates the macroscopic overview of the two series of DESS 3 weeks after transplantation. The DESS generated with a 3 day keratinocyte (3dK) pre-cultivation time (left panels) do not show a hydrophobic, dry cornified surface. In contrast, the skin analogs formed with a 6 day (6dK) keratinocyte pre-cultivation time show a cornified surface (center). Importantly 12 day (12dK) pre-cultivation of DESS did not improve the outcome: blood vessels were locally visible under the substitute (right panels, arrow) indicating a reduced epidermal thickness. Figure 4b statistically illustrates the measured size of the substitutes, pictured in Fig. 4a.

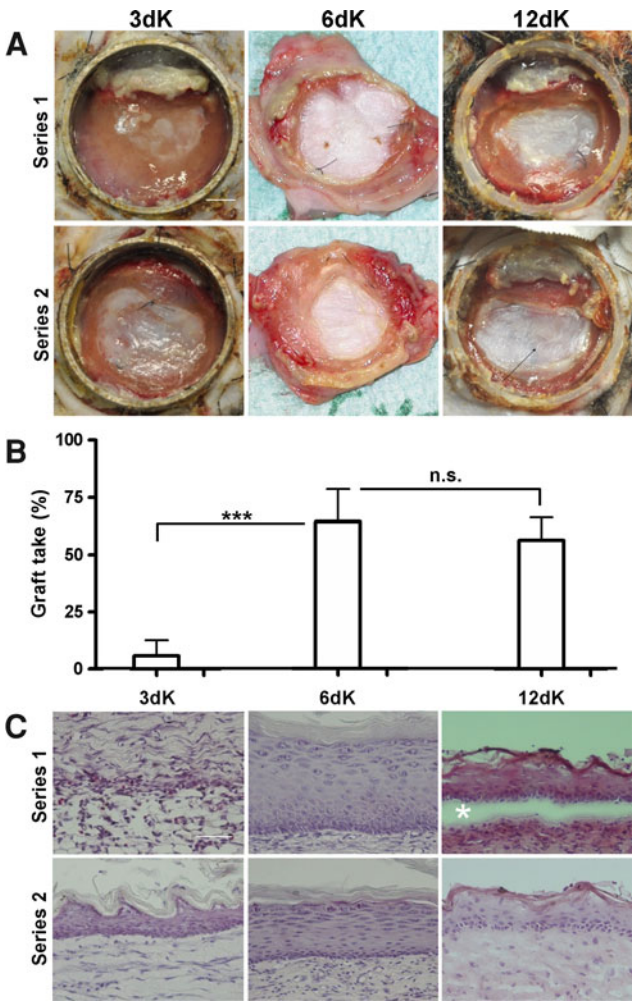

Fig. 4 Variation of the incubation time of keratinocytes on the dermal substitutes before transplantation: effect on graft take and stratification in vivo. **a** Macroscopic view of the grafts 3 weeks after transplantation. Both series of substitutes (refer to Table 1) are shown. *Scale bar* 5 mm. **b** Size of the transplanted DESS 3 weeks after grafting. Both series are included in the mean (mean ± SD, $n = 6$). ***$p < 0.0001$, *ns* not significant. **c** Eosin/Hematoxylin-stained paraffin sections of 3dK, 6dK, and 12dK-DESS 3 weeks after transplantation. The *asterisk* in 12dK of series 1 indicates a technical artifact. *Scale bar* 50 μm

H/E staining of 3dK confirmed the presence of a thin epidermal coverage in some areas, while in other areas no epidermis was found (Fig. 4c, left). 6dK produced a stratum corneum and numerous keratinocyte layers (middle panels). 12dK showed a similar result, yet the thickness of the epidermis was reduced (right).

Relevance of the air/liquid interface cultivation for transplantation

The second set of 6dK and 12dK was raised to the air–liquid interface 3 days before transplantation ($6dK^{air}$ and $12dK^{air}$ in Fig. 1c).

The graft size obtained from 6dk and $6dK^{air}$, respectively, was similar in the first experimental series (75 ± 2 vs. 74 ± 3 %) and slightly different in the second (52 ± 5 vs. 78 ± 1 %, $p = 0.0188$) (Fig. 5a). The epidermal thickness was generally reduced in $6dK^{air}$ (192 ± 18 vs. 165 ± 38 µm, not statistically significant) but the cornification was more pronounced (38 ± 11 to 73 ± 27 µm, $p = 0.0001$) (microscopic view in Fig. 5b, and statistical analysis in Fig. 5c).

The graft sizes obtained from 12dk and $12dK^{air}$ were different: 52 ± 7 vs. 96 ± 3 % ($p = 0.0001$) in the first series and 64 ± 7 vs. 88 ± 4 % ($p = 0.0013$) in the second (Fig. 5d). The epidermal thickness was similar in 12dK and $12dK^{air}$ (88 ± 3 vs. 77 ± 11 µm, not statistically significant, Fig. 5f compares 5d) but again, the cornification was more pronounced in $12dK^{air}$ (27 ± 6 vs. 95 ± 4 µm, $p = 0.0001$) (microscopic view in Fig. 5e, and statistical analysis in Fig. 5f).

Stratification markers in air-exposed DESS

Immunofluorescence analysis of the expression pattern of stratification markers confirmed the high quality of both air-exposed and non-air exposed DESS. No difference in the expression pattern of K1/involucrin (Fig. 6a), occludin/integrin-a6 (Fig. 6b), and K15/K19 (Fig. 6c) is apparent.

Discussion

The principal goal of this study was to test whether the culture time for human cell-derived DESS can be significantly reduced when compared to standard procedures commonly used in our laboratory. The findings obtained clearly demonstrate that it is possible to markedly reduce the current culture time from 21 to 12 days. Hereby, the main gain was attributable to a significant shortening of the incubation time of keratinocytes on the dermal template before transplantation, i.e. from 14 to 6 days.

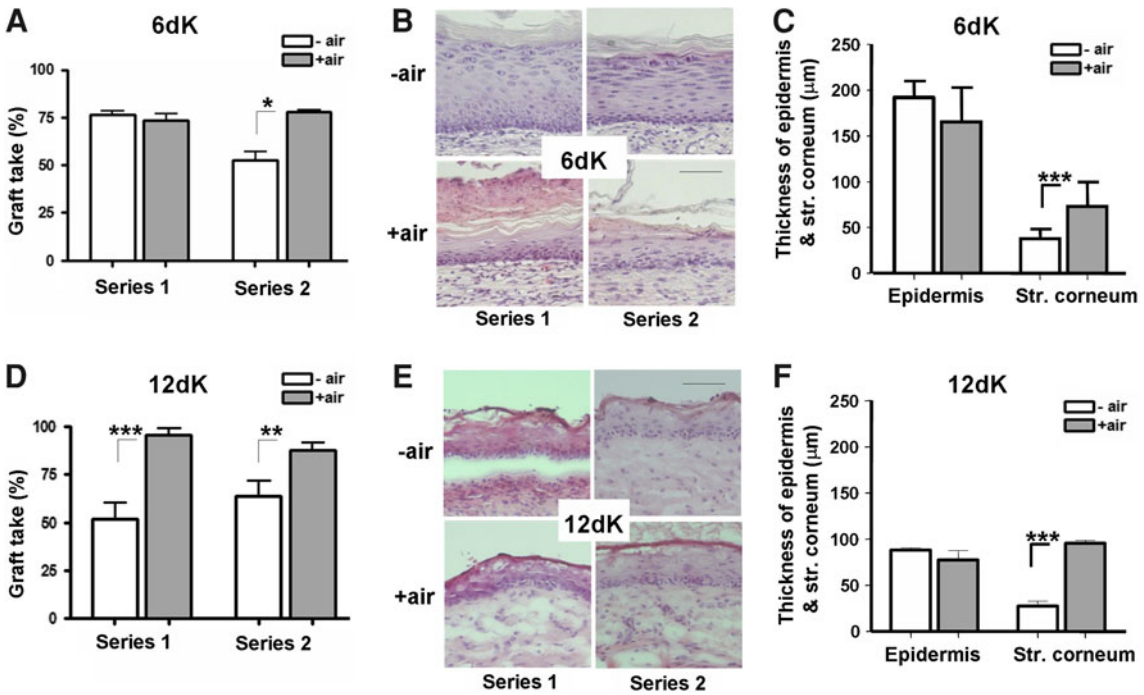

Fig. 5 Effect of air–liquid interface cultivation of DESS on graft take and stratification in vivo. **a, d** Size of the 6dK and 12dK (*white*) and $6dK^{+air}$ and $12dK^{+air}$ (*grey*) grafts 3 weeks after transplantation (mean ± SD, $n = 3$). Both experimental series are shown. **b, e** Eosin/Hematoxylin-stained paraffin sections of air-exposed (+air) and non-air exposed (−air) 6dK and 12dK 3 weeks after transplantation. *Scale bar* = 50 µm. **c, f** Thickness of the obtained air-exposed and non-air exposed 6dK and 12dK in µm (entire epidermis and stratum corneum only, mean ± SD, $n = 3$). $^{***}p < 0.0001$, $^{**}p < 0.0013$, $^{*}p < 0.0188$

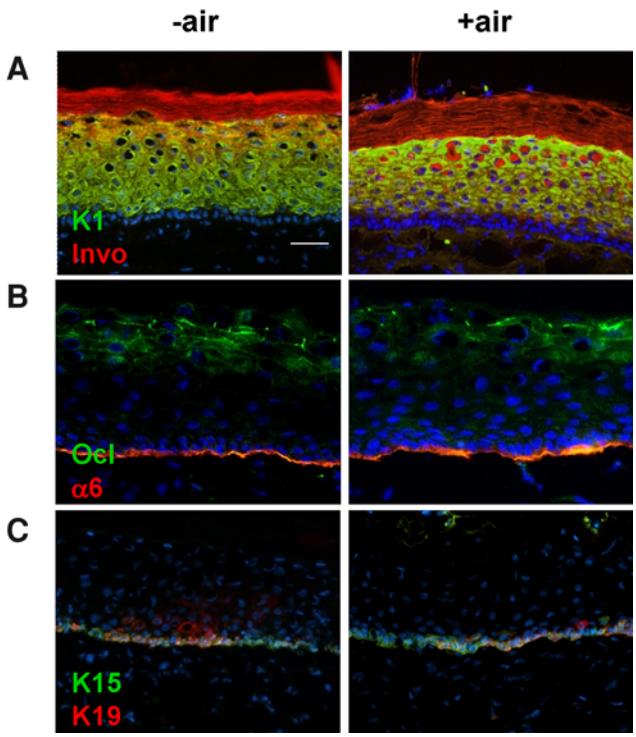

Fig. 6 Stratification markers in air-exposed 6dK-DESS. **a** Immunofluorescence double-staining of cryosections with antibodies against K1 (*green*) and involucrin (*red*), **b** occludin (*green*) and integrin α6 (*red*), or **c** K15 (*green*) and K19 (*red*). *Scale bar* for all *panels* 50 μm

How can this significant reduction of incubation time be explained? We speculate that the specific cell biology dynamics of keratinocytes in culture (as opposed to keratinocytes residing in their natural habitat) play a role. For a more detailed understanding, we provide a description of our current culture system and the key phenomena observed during DESS formation: once isolated and cultured, keratinocytes are seeded onto collagen hydrogels (=dermal templates). Then, keratinocytes start to proliferate and migrate horizontally to form a confluent layer on the top of the dermal template. Subsequently, the ongoing cell proliferation leads to formation of a multilayer that starts to stratify. Once the in vitro construct is raised to the air–liquid interphase, cornification gradually develops.

The above described processes typically last for 14 days. Thereafter, our constructs are transplanted and usually produce an anatomically and functionally near normal skin [9]. We also know that if DESS incubation time is significantly lengthened to e.g. 21 days, quality and transplantation results are still satisfactory. If, however, DESS incubation time is further prolonged to 28 days or more, then we increasingly observed degenerative features ultimately leading to massive cell death and complete loss of DESS viability.

In other words, the above mentioned evolution can roughly be described as an in vitro DESS life cycle comprising a "juvenile" (<14 days), a "mature" (14–21 days), and a "senile" phase (>21 days).

The findings presented here clearly indicate that "juvenile" and not only "mature" DESS already have the potential to induce near normal skin after transplantation, i.e. following only 6-day-long incubation time. Apparently, the 3–4 layers present at 6 days are sufficient to generate a normal epidermis after transplantation, i.e. there is no need to use "mature" DESS for a successful transplantation.

In this respect, it was important to test the relevance of raising DESS to the air–liquid interface before transplantation. Air–liquid interface culturing is one of the most important improvements of the last decades in the production of skin analogs. It was shown to induce stratification and cornification of the newly formed epidermis [17]. Nevertheless, we demonstrate here that for the purpose of transplantation this "maturation" step may be skipped, presumably for the same reasons as mentioned before.

To our knowledge, there is no information available regarding significantly shortened culture times for similar skin substitutes. The product most closely related to our DESS, the commercially available Apligraf®, uses allogeneic fibroblasts and keratinocytes. The allogeneic specification makes it possible to completely avoid any waiting time for the patient. Yet its production requires 20 days [18, 19].

Other approaches drastically reduce or even skip in vitro cultivation: starting from a small split-thickness skin biopsy, cell suspensions are applied onto the wound bed after 5 days of culturing or immediately after preparation (ReCell®) [20]. Yet, this strategy is definitely not appropriate to treat third degree burns and, generally speaking, its place in clinical practice is still controversial.

The last consideration regards the fibroblast pre-incubation time. We could only realize a minor gain of 1 day with the reduction of the fibroblast pre-incubation time before seeding keratinocytes. As a matter of fact, the minimal duration of this phase is critical for correct DESS development. The presence of fibroblasts in the dermal template of DESS has been shown to be crucially important [21–23]. Fibroblasts remodel the collagen hydrogel, prepare the formation of a basal lamina and the anchorage of epithelial cells, secrete growth factors, and finally sustain epidermal regeneration [24]. To fulfill these tasks, fibroblasts need a defined minimal amount of time. It is generally assumed that raising the number of fibroblasts in the gel would accelerate the organization of the dermal template [25], but this acceleration may also be accompanied by abundant production of granulation tissue and wound contraction, both of which are unwanted effects [26]. Therefore, we believe that the fibroblast pre-incubation time cannot be significantly shortened.

In summary and conclusion, this appears to be the first article to describe a very substantial shortening of the production time for laboratory grown human skin substitutes from 21 to 12 days without an obvious loss of graft quality. Our study may have important clinical implications in that a marked reduction of waiting time until autologous skin substitutes are ready for transplantation can definitely improve the fate of burn patients.

Acknowledgments This work was financially supported by the EU-FP6 project EuroSTEC (soft tissue engineering for congenital birth defects in children, contract: LSHB-CT-2006-037409), by the EU-FP7 project EuroSkinGraft (FP7/2007-2013: grant agreement No. 279024), by the EU-FP7 (MultiTERM, grant agreement No. 238551), and by the University of Zurich. We are particularly grateful to the Foundation Gaydoul and the sponsors of "DonaTissue" (Thérèse Meier, Robert Zingg) for their generous financial support and interest in our work.

Conflict of interest None.

References

1. Rheinwald JG, Green H (1975) Serial cultivation of strains of human epidermal keratinocytes: the formation of keratinizing colonies from single cells. Cell 6:331–343
2. O'Connor NE, Mulliken JB, Banks-Schlegel S, Kehinde O, Green H (1981) Grafting of burns with cultured epithelium prepared from autologous epidermal cells. Lancet 1:75–78
3. Gallico GG 3rd, O'Connor NE, Compton CC, Kehinde O, Green H (1984) Permanent coverage of large burn wounds with autologous cultured human epithelium. N Engl J Med 311:448–451
4. Munster AM (1996) Cultured skin for massive burns. A prospective, controlled trial. Ann Surg 224:372–375 discussion 375–377
5. Krupp S, Benathan M, Meuli M, Deglise B, Holzer E, Wiesner L, Delacretaz F, Chiolero R (1992) Current concepts in pediatric burn care: management of burn wounds with cultured epidermal autografts. Eur J Pediatr Surg 2:210–215
6. Cuono C, Langdon R, McGuire J (1986) Use of cultured epidermal autografts and dermal allografts as skin replacement after burn injury. Lancet 1:1123–1124
7. Boyce ST, Goretsky MJ, Greenhalgh DG, Kagan RJ, Rieman MT, Warden GD (1995) Comparative assessment of cultured skin substitutes and native skin autograft for treatment of full-thickness burns. Ann Surg 222:743–752
8. Gobet R, Raghunath M, Altermatt S, Meuli-Simmen C, Benathan M, Dietl A, Meuli M (1997) Efficacy of cultured epithelial autografts in pediatric burns and reconstructive surgery. Surgery 121:654–661
9. Biedermann T, Pontiggia L, Bottcher-Haberzeth S, Tharakan S, Braziulis E, Schiestl C, Meuli M, Reichmann E (2010) Human eccrine sweat gland cells can reconstitute a stratified epidermis. J Invest Dermatol 130:1996–2009
10. Costea DE, Loro LL, Dimba EA, Vintermyr OK, Johannessen AC (2003) Crucial effects of fibroblasts and keratinocyte growth factor on morphogenesis of reconstituted human oral epithelium. J Invest Dermatol 121:1479–1486
11. Pontiggia L, Biedermann T, Meuli M, Widmer D, Bottcher-Haberzeth S, Schiestl C, Schneider J, Braziulis E, Montano I, Meuli-Simmen C, Reichmann E (2009) Markers to evaluate the quality and self-renewing potential of engineered human skin substitutes in vitro and after transplantation. J Invest Dermatol 129:480–490
12. Braziulis E, Diezi M, Biedermann T, Pontiggia L, Schmucki M, Hartmann-Fritsch F, Luginbuhl J, Schiestl C, Meuli M, Reichmann E (2012) Modified plastic compression of collagen hydrogels provides an ideal matrix for clinically applicable skin substitutes. Tissue Eng Part C Methods 18(6):464–475
13. Armour AD, Powell HM, Boyce ST (2008) Fluorescein diacetate for determination of cell viability in tissue-engineered skin. Tissue Eng Part C Methods 14:89–96
14. Candi E, Schmidt R, Melino G (2005) The cornified envelope: a model of cell death in the skin. Nat Rev Mol Cell Biol 6:328–340
15. Stark HJ, Baur M, Breitkreutz D, Mirancea N, Fusenig NE (1999) Organotypic keratinocyte cocultures in defined medium with regular epidermal morphogenesis and differentiation. J Invest Dermatol 112:681–691
16. Borradori L, Sonnenberg A (1999) Structure and function of hemidesmosomes: more than simple adhesion complexes. J Invest Dermatol 112:411–418
17. Supp AP, Wickett RR, Swope VB, Harriger MD, Hoath SB, Boyce ST (1999) Incubation of cultured skin substitutes in reduced humidity promotes cornification in vitro and stable engraftment in athymic mice. Wound Repair Regen 7:226–237
18. Wilkins LM, Watson SR, Prosky SJ, Meunier SF, Parenteau NL (1994) Development of a bilayered living skin construct for clinical applications. Biotechnol Bioeng 43:747–756
19. Parenteau NL, Nolte CM, Bilbo P, Rosenberg M, Wilkins LM, Johnson EW, Watson S, Mason VS, Bell E (1991) Epidermis generated in vitro: practical considerations and applications. J Cell Biochem 45:245–251
20. Wood FM (2003) Clinical potential of autologous epithelial suspension. Wounds 15:16–22
21. Marionnet C, Pierrard C, Vioux-Chagnoleau C, Sok J, Asselineau D, Bernerd F (2006) Interactions between fibroblasts and keratinocytes in morphogenesis of dermal epidermal junction in a model of reconstructed skin. J Invest Dermatol 126:971–979
22. Nolte CJ, Oleson MA, Hansbrough JF, Morgan J, Greenleaf G, Wilkins L (1994) Ultrastructural features of composite skin cultures grafted onto athymic mice. J Anat 185(Pt 2):325–333
23. Okamoto E, Kitano Y (1993) Expression of basement membrane components in skin equivalents–influence of dermal fibroblasts. J Dermatol Sci 5:81–88
24. Auger FA, Rouabhia M, Goulet F, Berthod F, Moulin V, Germain L (1998) Tissue-engineered human skin substitutes developed from collagen-populated hydrated gels: clinical and fundamental applications. Med Biol Eng Comput 36:801–812
25. Stark HJ, Willhauck MJ, Mirancea N, Boehnke K, Nord I, Breitkreutz D, Pavesio A, Boukamp P, Fusenig NE (2004) Authentic fibroblast matrix in dermal equivalents normalises epidermal histogenesis and dermoepidermal junction in organotypic co-culture. Eur J Cell Biol 83:631–645
26. Lamme EN, Van Leeuwen RT, Brandsma K, Van Marle J, Middelkoop E (2000) Higher numbers of autologous fibroblasts in an artificial dermal substitute improve tissue regeneration and modulate scar tissue formation. J Pathol 190:595–603